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High-Yield Purification of Plasma Membranes from Transformed Human Keratinocytes in Culture

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The density pertubation technique with cationic silica microbeads was applied to prepare highly purified plasma membranes from cultured human keratinocytes. Trypsinized cells were coated successively with the beads (diameter ~50 nm, gravity > 2 g/cm^3) and polyacrylic acid before they were lysed by osmotic shock and mechanical shear. The plasma membranes remained in the form of large open sheets which could easily be separated from other cell organelles and the cytosol by low-speed centrifugation.

The membrane preparation was characterized by scanning and transmission electron microscopy, marker enzyme activities, one-dimensional sodium dodecyl sulfate polyacrymamide electrophoresis, and the specific beta-adrenergic receptor count. A yield of 79 ± 9% was calculated by comparing the amount of beta-adrenoceptors in the purified membrane preparation with that of a crude cellular particulate fraction. The specific beta-adrenoceptor count of these two preparations was 1.2 ± 0.02 and 0.2 ± 0.05 pmol/mg protein, respectively, indicating a 6-fold improved purification with this microbead technique. The purified membranes were essentially free from contamination of other cell organelles.

Evidence has accumulated that plasma membrane-associated events play an important role in the control of epidermal cell growth and differentiation. Drug-, diet-, and disease-related processes are supposed to modify, for example, membrane receptor binding characteristics and the expression of their function by changing the membrane microenvironment. Examples of this include the beta-adrenoceptor antagonists, glucocorticoids, and dietary essential fatty acids.

Up to the present time the study of these events was limited by the difficulty in obtaining plasma membranes from epidermal cells in sufficiently high yield and purity within a reasonable period of time. In order to overcome these difficulties we have examined the suitability of the density pertubation technique with cationic silica microbeads having a sp gr of 2 g/cm^3 and a diameter of about 50 nm. This technique has already proved useful for the high-yield purification of plasma membranes from the slime mold *Dictyostelium discoideum* [1], the yeast *Saccharomyces cerevisiae* [2], and the plant *Beta vulgaris*, [3].

The principle of the method is based on the tight binding of the cationic microbeads to the cell surface which carries a negative net charge. Upon cell lysis, the plasma membranes remain in the form of large open sheets which can easily be isolated and purified by centrifugation since their density is greatly enhanced by the attached microbeads. To obtain optimal results the method has to be modified specifically for the cell type under investigation [1–3].

In this paper we describe the adaptation of the procedure for the purification of plasma membranes from the transformed human keratinocyte line SV-K14. The purification was confirmed by scanning and transmission electron microscopy, by the analysis of enzyme activities, and by one-dimensional sodium dodecyl sulfate (SDS) gel electrophoresis. The yield was calculated by using the beta-adrenergic receptors as a plasma membrane marker.

MATERIALS AND METHODS

Chemicals

Culture media and fetal calf serum (FCS) were from Flow Laboratories and GIBCO. Aprotinin and deoxyribonuclease were purchased from Boehringer, Mannheim. Metrizamide was delivered from Serva, and polyacrylic acid, M_r 90,000, was a product of Aldrich Chemicals. [^{3}H]Dihydroalprenolol was obtained from NEN-France. A 30% (w/v) stock solution of cationic silica microbeads was kindly provided by Dr. B. Jacobson (Department of Biochemistry, University of Massachusetts, Amherst).

Cells and Culture Conditions

The SV-40 transformed human foreskin keratinocyte line SV-K14 (a kind gift from Dr. B. Lane, Imperial Cancer Research Fund, London) was used after about 20 passages. The cells were grown at 37°C (5% CO_2 humidified atmosphere) in Dulbecco's modified Eagle's medium (containing 5.5 mM glucose and 4 mM glutamine) = Ham's medium F12 (DMEM/F12) (1:1) containing 100,000 units penicillin, 100 mg streptomycin, and 250 μg amphotericin B per liter of medium which was supplemented with 5–10% FCS depending on the batch.

Trypsination of the Cells

Early confluent cultures were used. The cells were washed twice with phosphate-buffered saline (PBS) before treating them for 8 min at 37°C with PBS containing 0.04% (w/v) trypsin and 1% (w/v) EDTA. The trypsination was stopped by the addition of FCS to a final concentration of 20% (v/v).

Membrane Preparation

The following steps were carried out at 4°C. The cells were collected by centrifugation (5 min, 400 *g*) and washed once with PBS. They were resuspended in coating buffer (25 mM sodium acetate, pH 5.0; 0.8 M sorbitol; 0.1 M NaCl) to a final cell titer of 3×10^6/ml. After 5 min, they were centrifuged for 5 min at 80 *g*. The resulting pellet was resuspended in the same volume of coating buffer containing 20 μl of the microbeads stock solution per ml. After 5 min, the cells were recentrifuged (5 min, 80 *g*). They were washed once to remove the excess microbeads and were resuspended in coating buffer, pH 5.0, containing 75 μg of the anionic polymer polyacrylic acid, M_r 90,000, per ml. After 5 min, the cells were centrifuged and washed with coating buffer. The supernatant was carefully aspirated.

Remains of the coating buffer were removed by washing the cells once with lysis buffer (5 mM Tris-HCl, pH 7.5; 1 mM ethylene glycol-bis (2 aminoethylether) N,N′-tetraacetic acid (EGTA); 1 mM dithioerythritol (DTE); 1 μg/ml aprotinin). Essentially no cell lysis occurred during this step. However, when the cells were resuspended in fresh

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Abbreviations:
DMEM: Dulbecco's modification of Eagle's medium containing 5.5 mM glucose and 4 mM glutamine
DTE: dithioerythritol
EGTA: ethylene glycol-bis (2 aminoethylether) N,N′-tetraacetic acid
FCS: fetal calf serum
F12: Ham's medium F12
PBS: phosphate-buffered saline without calcium or magnesium
SDS: sodium dodecyl sulfate

lysis buffer at a concentration of 1.5×10^6 cells/ml, lysis was complete after 5–10 min. The suspension was transferred into a tightly fitting glass homogenizer to rupture the nuclei with about 10–15 strokes.

To digest the liberated DNA which caused aggregation between the plasma membrane sheets and other cell organelles, the suspension was supplemented with $MgCl_2$ and deoxyribonuclease to a final concentration of 25 mM and 50 μg/ml, respectively. During a 15-min incubation time (at room temperature) a few strokes were added to get a homogeneous suspension which was centrifuged at 400 *g* for 5 min. The resulting plasma membrane pellet was washed twice in a larger volume of lysis buffer to remove the deoxyribonuclease and other contaminations.

For further purification, the plasma membranes were resuspended in lysis buffer and layered over a 1.5 cm-high metrizamide cushion, 815 g/liter, giving a density of about 1.45 g/cm^3. After 25 min at 82,700 *g* the membrane pellet was washed twice with lysis buffer to remove the metrizamide.

Beta-Adrenergic Receptor and Enzyme Assays

The binding assay experiments for the beta-adrenergic receptors were performed as described by Gazith et al [4]. Cytochrome c oxidase was measured according to Hodges and Leonard [5]. Glucose 6-phosphatase was assayed according to the procedure of Baginski et al [6]. Protein content was determined with the Bio-Rad method.

Electrophoresis

Proteins from whole cells after trypsinisation from a cytosolic supernatant (after 1-h centrifugation at 100,000 *g*), from the resulting pellet (particulate fraction), and from purified plasma membranes were separated on a 12.5% SDS polyacrylamide gel according to the procedure described by Laemmli [7]. They were stained with Coomassie blue.

Electron Microscopy

All samples were fixed by bringing suspensions up to 2.5% (w/w) glutaraldehyde and incubating them for 1 h at room temperature. Samples were then washed in PBS, dehydrated in graded alcohol, and dried in a Balzers critical point drier. They were gold-coated to a thickness of 6–8 nm in the sputtering attachment of the Balzers FDU 010 and examined with a JEOL SEM T300 at 20–30 kV.

For transmission electron microscopy the cells were fixed as described above and postfixed with 1% osmium tetroxide in PBS for 1 h at 4°C. Cells were dehydrated in a graded series of alcohol, placed in propylene dioxide, and embedded in Epon. Thin sectioning was performed with a Reichert ultracut and stained with uranyl acetate and lead citrate to be examined with a JEOL 1200EX.

RESULTS AND DISCUSSION

Coating of the Keratinocytes

Coating of the keratinocytes with microbeads was accomplished after a 5-min incubation of the cells in coating buffer containing the silica microbeads. It was important to have the coating buffer adjusted to a pH below 5.5 since at higher pH values the microbeads aggregated and precipitated.

Fig 1 shows trypsinized keratinocytes before (*A*) and after (*B*) coating with the microbeads. It is apparent that the whole cell surface becomes covered with the microbeads and that the high osmotic pressure of the coating buffer causes the cells to shrink. After removing the excess microbeads, the cells were incubated for a short time with the anionic polymer polyacrylic acid in order to stabilize the microbead pellicle [1].

Cell Lysis and Purification of Plasma Membrane

Cell lysis was accomplished after resuspending the cells in lysis buffer at a concentration of 1.5×10^6/ml. The pH of the lysis buffer was adjusted to 7.5 to avoid binding of intracellular contaminating material to the plasma membranes. EGTA was added to trap divalent cations and thereby ease cell lysis.

Most of the nuclei remained intact after this treatment. Owing to their high density, this led to a cosedimentation with the plasma membranes. Therefore, the homogenate was transferred into a tightly fitting glass homogenizer to burst the nuclei with 10–15 strokes.

Since this treatment liberated DNA, which caused considerable aggregation between the plasma membrane sheets, deoxyribonuclease was added to the homogenate. A 15-min incubation was enough to retain a homogeneous suspension.

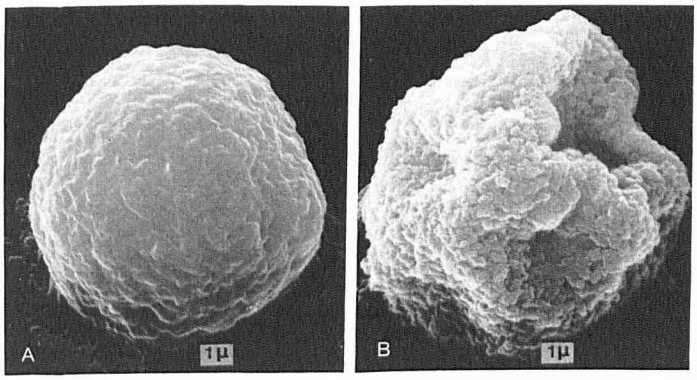

FIG 1. Scanning electron microscopy of a human keratinocyte (SV-K14) after trypsinization, (*A*) in PBS buffer and (*B*) coated with microbeads in the hyperosmotic coating buffer.

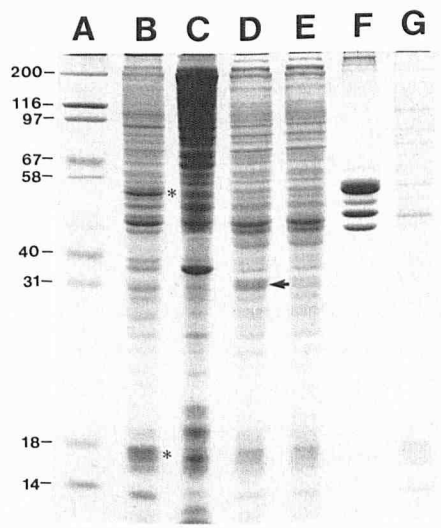

FIG 2. SDS polyacrylamide gel electrophoresis in a 12.5% gel of a total protein extract from trypsinized human keratinocytes (*lane B*), proteins extracted from purified plasma membranes (*lane C*), cytosolic proteins from a 100,000 *g* supernatant with (*lane D*) and without (*lane E*) DNase, SV-K-14 keratins (*lane F*), extracted as described by Winter et al [8] and the proteins from the particulate fraction (*lane G*). The *asterisks* indicate 53Kd keratins and 14–16Kd histones, the *arrow* in lane D marks the DNase. *Lane A* shows the position of the molecular weight marker proteins.

The coated plasma membrane sheets already sedimented at 400 *g* for 5 min and could now be purified by successive washings in lysis buffer.

A further purification was achieved by spinning the washed plasma membranes through a metrizamide cushion with a density of D = 1.45 g/cm^3. This step was especially necessary when the cell population contained dead cells which did not lyse upon the osmotic shock procedure. The dead cells banded on top of the cushion and were thus separated from the plasma membranes.

Preliminary experiments using this technique for the isolation of plasma membranes from nontransformed human keratinocytes in culture proved its general applicability. However, problems arose from the heterogeneity of the cell population. Experiments are in progress to separate differentiating and differentiated cells from those with basal cell character by an additional centrifugation preceding the coating procedure.

Electrophoretic Analysis

Comparing the one-dimensional electrophoretic pattern of the protein extract from whole cells with that from purified plasma membranes (Fig 2) the following statements can be

made: (i) the characteristic 53Kd keratin band, component 7,8 according to the cytokeratin catalog [9], observed in the whole cell extracts (upper asterisk) is absent in the purified plasma membranes, (ii) the large amount of 14–16Kd histones (lower asterisk) almost disappear during the purification. These findings indicate that our membrane preparation is essentially free from cytoskeleton and nuclei contaminations.

Marker Enzymes and Beta-Adrenergic Receptors

The purity of the preparation was further verified by the assay of marker enzymes. We restricted ourselves to determining cytochrome c oxidase activity (a mitochondrial marker) and glucose 6-phosphatase (a marker for the endoplasmatic reticulum) for two reasons: (i) The specificity of marker enzymes generally used to detect contaminations after subcellular fractionation is not yet well established for human keratinocytes, and (ii) the pelleting forces reported for the different cell organelles are at least one order of magnitude higher than those used for our purification [10].

No cytochrome c oxidase activity was found in the purified plasma membranes indicating the absence of mitochondrial material. However, 3% of the total glucose 6-phosphatase activity was recovered in the membrane preparation. This indicates that a part of the endoplasmatic reticulum remained bound to the plasma membrane.

The presence of beta-adrenergic receptors in the plasma membrane of human keratinocytes is well established [4] and was therefore used to evaluate the yield after purification. Before doing so it was essential to prove that no receptor sites became masked by coating the cell surface with the microbeads. We therefore disrupted microbead-coated and noncoated cells with the Parr bomb as described [4]. The homogenates were centrifuged for 60 min at 100,000 *g* to recover the particulate fraction of the cells. The yield of protein and the specific count of beta-receptors (0.2 ± 0.05 pmol/mg protein) was the same for both preparations.

By setting the amount of beta-adrenoceptors obtained with this procedure to 100% we calculated the yield for the purified membranes. The yield determined from 15 independent experiments was 79 ± 9% and the specific beta-adrenergic count was 1.2 ± 0.02 pmol/mg protein.

Electron Microscopy

Scanning electron microscopy showed that the plasma membranes remained in the form of large sheets throughout the purification procedure (Fig 3*A*). Fig 3*B* demonstrates that only the outside of the plasma membrane is covered with microbeads. The smooth inner side of the membrane which in this picture is folded over the outside is clearly visible.

Thin sections of purified plasma membranes (Fig 4) confirmed that our preparations were essentially free of contamination by other cell organelles; some of the smaller plasma membrane sheets seemed to have formed vesicles.

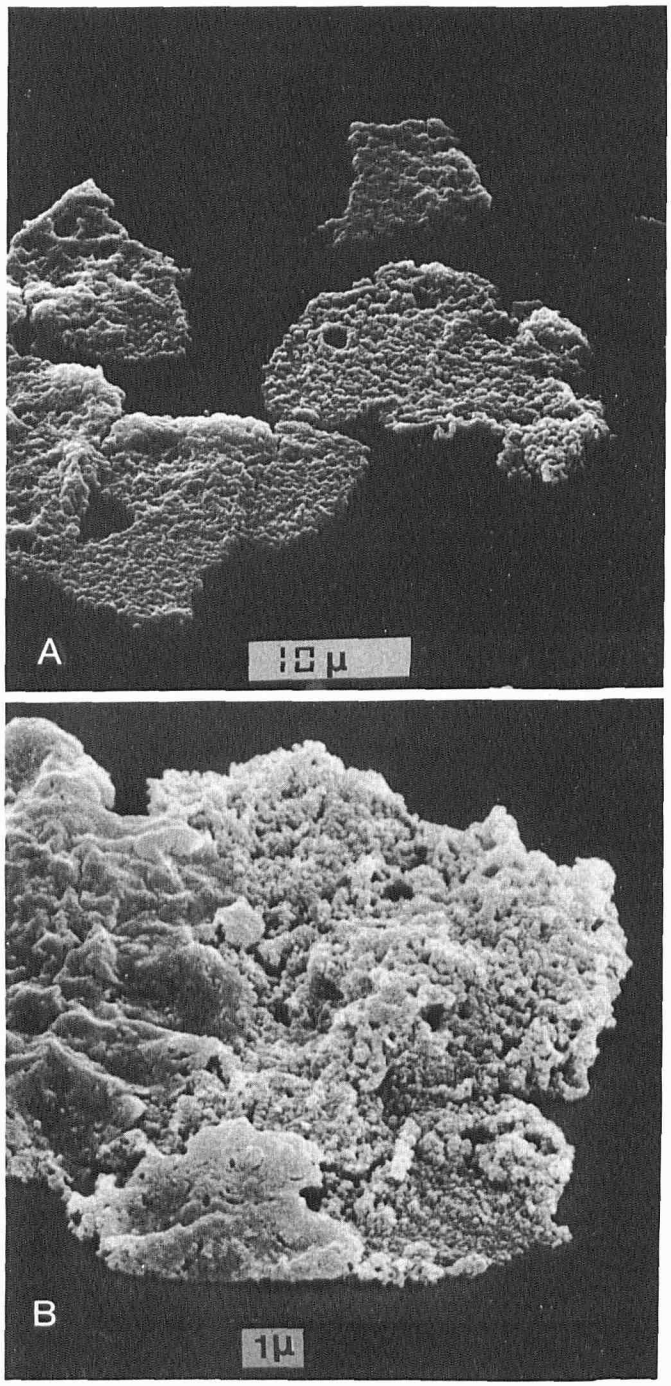

FIG 3. Scanning electron microscopy of (*A*) microbead-coated plasma membranes after their purification, and (*B*) a plasma membrane sheet where the smooth inner side of the membrane is folded over the microbead-coated outside.

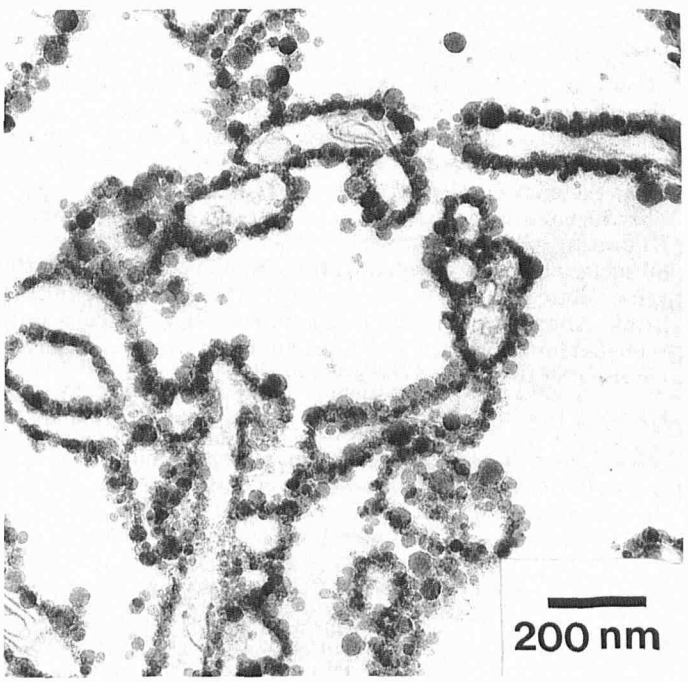

FIG 4. Transmission electron microscopy of ultrathin-sections of purified plasma membranes.

Conclusions

The purification procedure described in this paper has the advantage over conventional techniques in that within a short time and under mild conditions a high yield of plasma membranes can be obtained. The membranes remain mainly in the form of large sheets preserving natural characteristics as, e.g., their property to specifically bind beta-adrenergic ligands.

Owing to its rapidity and high degree of purification this method should be especially suited to study drug-induced changes in the lipid and protein composition at the plasma membrane level.

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